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Review

Strategies to establish the link between biosynthetic gene clusters and secondary metabolites

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ABSTRACT

Filamentous fungi produce a vast number of bioactive secondary metabolites (SMs), some of which have found applications in the pharmaceutical industry including as antibiotics and immunosuppressants. As more and more species are whole genome sequenced the number of predicted clusters of genes for SM biosynthesis is ever increasing – holding a promise of novel useful bioactive SMs. To be able to fully utilize the potential of novel SMs, it is necessary to link the SM and the genes responsible for producing it. This can be challenging, but many strategies and tools have been developed for this purpose. Here we provide an overview of the methods used to establish the link between SM and biosynthetic gene cluster (BGC) and vice versa, along with the challenges and advantages of each of the methods. Part I of the review, associating BCG with SM, is divided into gene manipulations native strain and heterologous expression strategies, depending on the fungal species. Part II, associating SM with BGC, is divided into three main approaches: (1) homology search (2) retro-biosynthesis and (3) comparative genomics.

1. Introduction

Fungi are an extraordinary source of bioactive secondary metabolites (SMs) including both medically utilized and novel unexplored molecules. The best known exploited fungal bioactive SMs include antibiotics such as penicillin and cephalosporin, hypercholesterolaemic agents such as lovastatin, immunosuppressants such as cyclosporin and mycophenolic acid, as well as antifungals such as echinocandin and its derivatives (Keller et al., 2005; Hautbergue et al., 2018; Regueira et al., 2011). Besides SMs with beneficial properties, fungal SMs also includes harmful toxins. Highly carcinogenic aflatoxins are produced by members of the genus *Aspergillus* (Bennett and Klich, 2003). Other toxins that are detrimental to human, animal and plant health include fumonisins, ochratoxin and gliotoxin (Bennett and Klich, 2003; Kwon-Chung and Sugui, 2009). The natural biological functions of SMs can range from defensive weaponry against competitors or predators, offensive virulence factors in infecting/pathogenic fungi, agents of symbiosis, metal transporting agents, to hormones and differentiation effectors (Demain and Fang, 2000).

The genes responsible for producing the SMs are often arranged in clusters on the genome, referred to as biosynthetic gene clusters (BGCs). Such clusters typically contain genes encoding one or more enzymes that synthesize the core structure of the compound (backbone enzymes), tailoring enzymes modifying the core structure, and potentially regulatory proteins such as transcription factors (TFs) and resistance genes encoding e.g. transporters (Osbourn, 2010; Brakhage, 2013). The most common backbone enzymes are polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs). Due to this and the arrangement of the genes in clusters, it is possible to predict BGCs in fungal genomes using predicted sequence domains and knowledge-based algorithms such as SMURF and antiSMASH (Khaldi et al., 2010; Blin et al., 2017). There are however also BGCs that are difficult to predict using these algorithms since they do not follow the basic conventions. Examples include tryptoquivaline in *Aspergillus clavatus* (Gao et al., 2011) and the echinocandin in *Emericella rugulosa* (Cacho et al., 2012), which are split in two parts. In addition, some cluster types are not easily detected by the prediction algorithms such as the terpenoid-based clusters since the amino acid sequences of terpene synthases are not as conserved as PKSs and NRPSs (Keller et al., 2005; Khaldi et al., 2010). As the prediction algorithms do not readily identify them, additional manual genome mining needs to be performed to identify them as demonstrated by Bromann et al. (2012). Other algorithms identifying BGCs include ClusterFinder (Cimermancic et al., 2014) and co-expression based methods (Andersen et al., 2012; Vesth et al., 2016). Weber and Kim have recently created an excellent portal summarizing bioinformatic tools and methods used in identification and analysis of BGCs

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(Weber and Kim, 2016).

With the sequencing of the first fungal genomes, one of the major discoveries was that the number of BGCs greatly outnumbered the known SMs, thereby revealing an even bigger SM repertoire in these species but also showing that most BGCs are not active or active enough to produce a detectable amount of SM during standard laboratory cultivation (Keller et al., 2005; Nierman et al., 2005; Payne et al., 2006; Schneider et al., 2008). This indicated that there was a substantial hidden potential of both novel and potentially useful bioactive SMs. To fully understand and exploit the rich resource of fungal SMs, it is important to establish the link between the SM and the BGC, both to be able to discover novel SMs and potentially to set the stage for industrial production of SMs as it allows process optimization by metabolic engineering and/or heterologous expression.

Here we present an overview of various strategies that can be used to discover the connection between a SM and BGC. The aim of this review is to give an overview of strategies that have been used to study secondary metabolism in fungi, illustrated by selected examples. This review consists of two main parts: (Part I) Associating a biosynthetic gene cluster with the secondary metabolite and (Part II) Associating a secondary metabolite with the biosynthetic gene cluster. Part I is divided into two principal strategies: native and heterologous strategies depending on the host. These are further divided into strategies for silent and active clusters for native hosts, while the heterologous expression strategies are divided into selection of hosts and design of gene constructs. Part II is divided into three sections depending on the approach: (1) homology search (2) retro-biosynthesis and (3) comparative genomics.

2. Part I: Strategies associating a biosynthetic gene cluster with the secondary metabolite it produces

The aim of the strategies in presented in Part I is to elucidate the biosynthetic pathway and identify the secondary metabolite (SM) produced by a specific biosynthetic gene cluster (BGC). The starting point is thus a putative BGC. The clusters are often selected by researchers for further analysis either based on similarity to a cluster responsible for the production of an important/known compound or because it has completely new characteristics. Once a cluster of interest has been identified, there are several ways of elucidating the molecule(s) it can synthesise; which method to use depends on various circumstances. An overview of the key questions to clarify and strategies is outlined in Fig. 1. The first key question (Q1 Fig. 1) is whether the cluster is found in a cultivatable fungus. If this is not the case then heterologous expression has to be used. If the fungus is cultivatable, the next question (Q2 Fig. 1) is whether it is amenable to genetic engineering; if the answer is negative again then heterologous expression must be used. If the answer is affirmative, strategies in the native host can be used. These strategies are divided based on the expression of the cluster. The last question (Q3 Fig. 1) is thus whether the cluster is actively expressed. If the cluster is silent, there are various ways of activating the cluster both based on external factors and genetic engineering strategies. If the cluster is active, or have been activated, gene deletion strategies can be used. Each of the mentioned strategies are explained and discussed in detail in following sections. An overview of the various strategies and their advantages and disadvantages can be seen in Table 1. The examination of a BGC is usually not a single process and the best studies requires a combination of various strategies to clarify the link between SM and BGC and to elucidate all the steps in the biosynthetic pathway.

It should be noted that the successfulness of the strategies mentioned here are highly sensitive to the methods used for chemical extraction and isolation. The SM of interest needs to be detectable with the extraction method used and isolated in amounts above the detection limit of the instrument. It is therefore essential to select which methods to use depending on the SM of interest. This is an important consideration but will not be covered further in this review; for more information refer to the following reviews (Luzzatto-Knaan et al., 2015; Nielsen and Larsen, 2015).

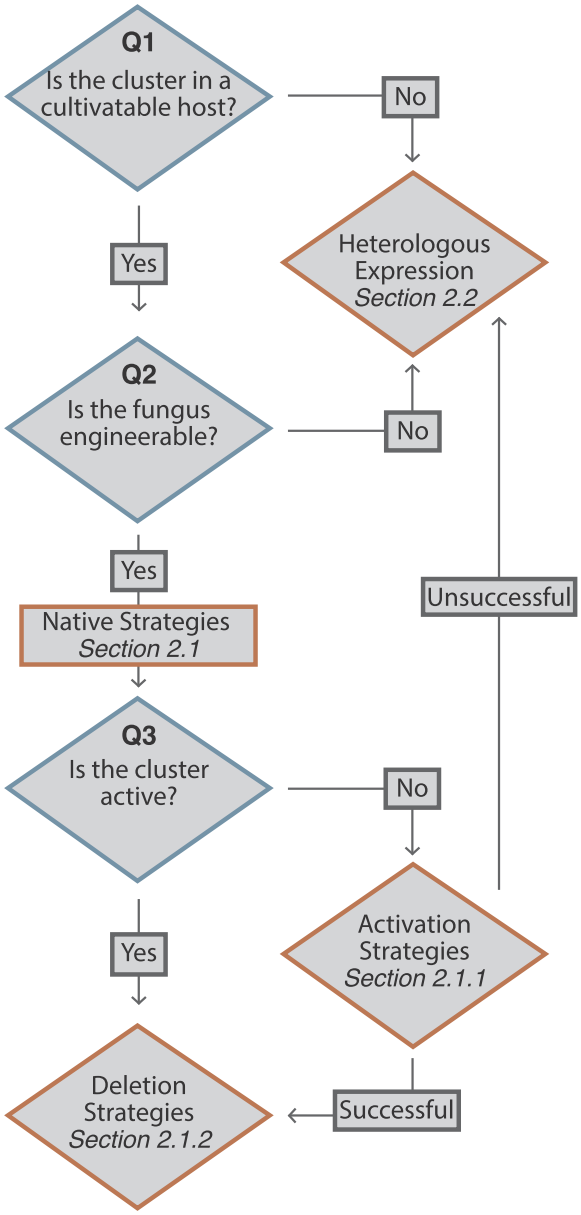

Fig. 1. Flow diagram of key questions to ask determining which strategy to use when starting from a cluster of interest. Questions are outlined in turquoise and the strategies are outlined in orange.

2.1. Strategies in native hosts

If a BGC is found in a cultivable and genetic engineerable species, it is possible to investigate the BGC directly in the native host. The prerequisite for this is that the BGC is transcriptionally active or that it is possible to activate it in the laboratory, in many cases this is not a trivial task. One method of investigating if a cluster is active could be to perform RT-PCR analysis to see if the genes are transcribed at a certain condition which could give an indication of the production of the SM of interest (Schroeckh et al., 2009; Gallo et al., 2014). Below, we review different strategies to activate BGCs ranging from simple to more complex. This is followed by a section outlining gene deletion strategies to demonstrate the link between BGC and SM synthesis and to dissect the individual functions of the genes in the BGC towards formation of

Table 1
Overview of strategies used in the association of biosynthetic gene clusters with the produced secondary metabolite along with the advantages and disadvantages for each strategy.

Strategy	Advantages	Disadvantage
	Strategies activating transcriptionally silent gene clusters	
Environmental stimuli	– Simple method – Not requiring molecular tools – Can be used on unexplored species	– Alters expression of many clusters – Not specific – Many conditions need to be tested
Global regulator	– Relatively easy way of changing the expression of secondary metabolites	– Affecting many clusters and hence not specific – Requires genetic modifications
Chromatin remodelling factors	– Relatively easy way of changing expression – Does not require genetic modifications	– Affecting many clusters and hence not specific
Cluster specific Transcription Factor (TF)	– Specific targeting the cluster of interest – Only manipulation of one gene is required – Coordinated expression of entire cluster of interest	– Requires genetic modifications – Requires a cluster with a TF
Promoter exchange	– Specific for the cluster of interest – Does not require a TF – Can be used on all clusters	– Requires genetic modification – Labor intensive
	Strategies for transcriptionally active gene clusters	
Gene deletions	– Provides direct link between gene and production of secondary metabolite	– Requires an expressed cluster – Requires molecular tools
Gene deletion libraries	– Provides direct link between gene and production of secondary metabolite – Allows larger scale investigations	– Requires an expressed cluster – Requires molecular tools – Labor intensive

the SM.

2.1.1. Strategies for triggering activation of silent biosynthetic gene clusters

The BGCs that are not expressed during standard conditions are often referred to as silent. Since the discovery of the large number of silent and unexplored clusters, efforts have gone into developing strategies for activating these silent gene clusters, which were reviewed by Brakhage and Schroeckh (2011). The strategies include global changes such as modified growth conditions, global regulators and chromatin remodelling plus cluster-specific strategies such as TF overexpression and promoter replacement, which are outlined in the following sections and in Fig. 2 S1-S4.

Environmental stimuli. The function in the natural niche of SMs are often not entirely clear, but naturally BGCs are induced at specific conditions to cope with certain environments. For instance some SM have been shown to fight competitors, attack host systems, communicate with collaborators, or adjust to stress conditions such as starvation or high salt concentrations and are synthesised under those triggers (Fox and Howlett, 2008). These natural regulation mechanisms can be used to activate gene clusters*in vitro*by changing the growth conditions mimicking various environments by different media compositions and other environmental conditions such as temperature or pH (Fig. 2S1).

The approach of systematically varying cultivation parameters to increase and investigate the secondary metabolism of an organism was termed 'OSMAC' (One Strain; Many Compounds) by Bode et al. and used to increase the number of compounds identified from six bacteria and fungal species, thus identifying more than 100 compounds belonging to 25 structural classes (Bode et al., 2002). Similarly, aspoquinolones AD was discovered from *A. nidulans* by investigation of the metabolome of *Aspergillus nidulans* grown at 40 different culture conditions (Scherlach and Hertweck, 2006). The secondary metabolism of *Aspergillus fumigatus* was also investigated varying the media composition and temperature (Frisvad et al., 2009).

Gressler et al. used a combination of metabolic profiling, monitoring gene expression, and a *lacZ* reporter strain to identify on which media a BGC of interest from *Aspergillus terreus* was active (Gressler et al., 2011). This strategy led to the identification of isoflavipucine and dihydroisoflavipucine as the SMs produced by a BGC. Ionic liquids have been shown to stimulate SM production in *A. nidulans* where 32% of described backbone genes were up-regulated, whereas normally silent*in vitro*(Alves et al., 2016). A number of studies have been made of the aflatoxin/sterigmatocystin production in *Aspergillus* spp. showing that environmental conditions like pH (Keller et al., 1997), nitrate (Leonard, 1998) and temperature (OBrian et al., 2007) affect the production. Pseurotin A biosynthesis in *A. fumigatus* is activated by hypoxia (Vödisch et al., 2011) and the secondary metabolism in *Fusarium fujikuroi* has been shown to be affected by nitrogen sources (Teichert et al., 2006, 2008).

Co-cultivation of fungal species with bacteria is another environmental stimulus that has been used successfully to activate silent gene clusters. Schroeckh et al. cocultivated *A. nidulans* with a collection of 58 soil-dwelling actinomycetes and showed fungal-bacterial interaction lead to the activation of specific fungal secondary metabolism genes including genes involved in the biosynthesis of orsellinic acid (Schroeckh et al., 2009). Wakefield et al. grew *A. fumigatus* strain MR2012 with isolates of *Streptomyces leeuwenhoekii* which led to the production and identification of luteoride D (a luteoride derivative) and pseurotin G (a pseurotin derivative) plus production of SMs not previously identified in this species (terezine D and 11-O-methylpseurotin A) (Wakefield et al., 2017).

Global regulators of secondary metabolism. Global regulators are proteins affecting the expression of several BGCs orchestrating a coordinated response to environmental factors (Fig. 2 S3). The concept of global regulators of SMs was introduced by Bok et al. with the investigation of the LaeA protein and its effects on SM production in several *Aspergillus* species (Bok and Keller, 2004). In *A. nidulans* the production of sterigmatocystin and penicillin decreased in a *laeA* deletion strain and a similar pattern was seen for *A. fumigatus laeA* mutants where the production of gliotoxin decreased and overexpression of *laeA* in *A. terreus* increased the production of lovastatin. LaeA was thus established as a global regulator of secondary metabolism in *Aspergillus* species (Bok and Keller, 2004). In a following study, *laeA* deletion and overexpression strains were used to identify active clusters through expression analysis in *A. nidulans*. A gene deletion in one of the clusters revealed that the cluster is responsible for producing terrequinone A (Bok et al., 2006). The study of LaeA has been expanded to many other filamentous Ascomycetes where the link between LaeA and secondary metabolite production has been established, for instance in the synthesis of T-toxin in *Cochliobolus heterostrophus* (Bi et al., 2013) and bikaverin, fumonisins, fusaric acid and fusarins in *Fusarium verticillioides* (Butchko et al., 2012), just to mention a few. For an excellent review of this topic, please refer to Jain and Keller (2013).

LaeA is part of the velvet complex with VeA and VelB, which connects light-response, developmental regulation and regulation of

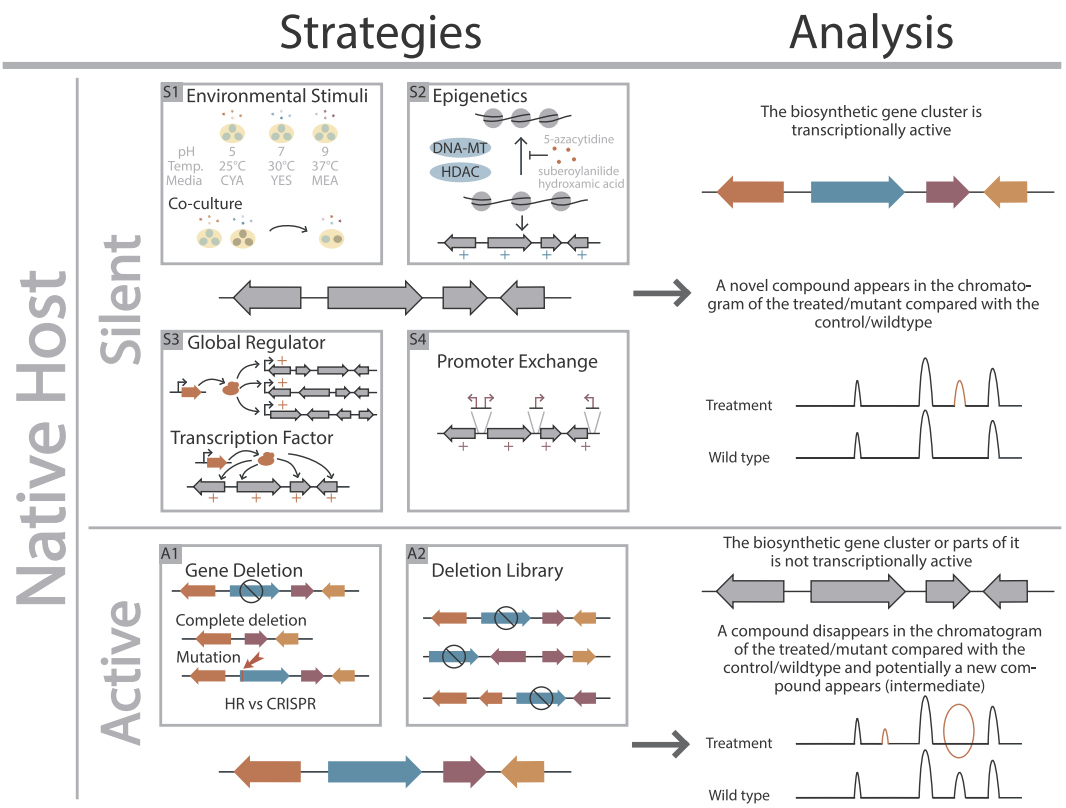

Fig. 2. Strategies for investigating a cluster in the native host. At the top are the strategies useful for activating silent gene clusters. S1 – Environmental stimuli changing pH, temperature, media components or co-culture with other microorganisms, S2 – Epigenetics or chromatin remodelling; either by adding compounds manipulating the epigenome or by gene deletions of epigenetic regulators, S3 – Overexpression of a global or cluster-specific transcription factor, S4 – promoter exchange of all the biosynthetic genes. Ideally these strategies leads to the activation of the gene cluster and the production of new compounds in the chemical analysis. In the bottom part are the strategies useful for active (expressed) biosynthetic gene clusters. A1 – gene deletion, A2 – the generation of a gene deletion library. These strategies leads to changes in the chromatogram often by the disappearance of the final compound and potentially the appearance of intermediates.

secondary metabolism (Bayram et al., 2008). A study comparing the transcriptional profile of an *A. fumigatus* wild type, *laeA* deletion and a complementation control strain found that 13 out of 22 BGCs were positively regulated by LaeA on a transcriptional level (Perrin et al., 2007). Of the LaeA-regulated clusters, 54% are located within 300 kb of telomeres. This could suggest a relationship between LaeA activity and chromatin modification that was hypothesized by Keller et al. (2005); however the hypothesis that LaeA methylates histones has not yet been verified nor refuted (Jain and Keller, 2013).

Several other proteins have general regulatory functions affecting secondary metabolism. These includes the nitrogen regulator AreA (Janevska and Tudzynski, 2018; Mihlan et al., 2003; Wiemann et al., 2013), the pH regulator PacC (Espeso and Peñalva, 1996), and the carbon regulator CreA (Dowzer and Kelly, 1991; Esperón et al., 2014). Recently two additional global regulators of secondary metabolism, MjkA and MjkB, have been identified using co-expression networks in *A. niger* (Schäpe et al., 2019). In addition to this, many specialized regulators exist, for instance the AtfA/AtfB regulators for the synthesis of aflatoxin (Gressler et al., 2015; Wee et al., 2017).

Chromatin remodelling factors. As mentioned in connection with LaeA, chromatin remodelling has been shown to be involved in regulation of secondary metabolites (SMs). The relationship between SM and chromatin structure can be exploited to activate otherwise silent gene clusters. The epigenetic landscape can both be modified by gene manipulation methods e.g. gene deletion or chemically by adding small molecules manipulating the fungal epigenome (Fig. 2 S2).

The *hdaA* gene encoding a histone deacetylase (HDAC) was deleted in *A. nidulans* causing an increase in the levels of the two sub-telomeric cluster products, penicillin and sterigmatocystin, while the non sub-telomeric cluster product terraquinone A levels were unaffected (Shwab et al., 2007). To investigate the mechanism in other fungi, *Alternaria alternata* and *Penicillium expansum* were treated with a HDAC inhibitor (Trichostatin A), which resulted in increased levels of several SMs in both species. This study showed that HdaA plays an important role in suppression of SM located in the sub-telomeric regions and that it might be a conserved mechanism across fungal species (Shwab et al., 2007).

Williams et al. showed that small-molecule epigenetic modifiers were effective in 11 out of 12 fungal species tested in that one or more modifiers caused the production of new SMs or enhanced production of known SMs compared to untreated controls. Two species were investigated further, *Cladosporium cladosporioides* and *Diatrype disciformis*, and new SMs (oxylipins and cladochromes from *C. cladosporioides* and lunalides from *D. disciformis*) were identified and characterised in each species showing the applicability of the method (Williams et al., 2008). In a study of *A. niger*, transcription of 36% of the predicted clusters in *A. niger* were significantly upregulated by the deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) (Fisch et al., 2009) illustrating the potential of chromatin remodelling in the activation of silent gene clusters. Again it was shown that the clusters located near the telomeric regions were mainly affected.

Besides addition of small molecules, deleting genes involved in chromatin remodelling impact SM production. In *A. nidulans*, deletion of the gene encoding a small ubiquitin-like protein SUMO involved in processes such as regulation of transcription, chromatin structure, and DNA repair increased the production of asperthecin while decreasing austinol/dehydroaustinol and sterigmatocystin production. This subsequently allowed for identification of the biosynthetic genes involved in asperthecin production (Szewczyk et al., 2008). CclA is a part of the complex COMPASS, an eukaryotic transcriptional effector methylating lysine 4 of histone H3 (H3K4) thus affecting chromatin-mediated

processes. Deletion of *cclA* in *A. nidulans* activated expression of otherwise silent BGC including the new cluster generating monodictyphenone, emodin and emodin derivatives, plus a cluster encoding two anti-osteoporosis polyketides, F9775A and F9775B (Bok et al., 2009). This study thereby showed that the deletion of genes involved in chromatin remodelling can affect secondary metabolism and can be used as a strategy to activate silent BGCs.

Cluster-specific transcription factors. In addition to global regulators of the expression of genes in BGCs, cluster-specific TFs also exist. These are located within the BGC and usually function as an activator of the specific BGC (Keller et al., 2005).

Overexpressing of the cluster-specific TFs can therefore be used as a targeted strategy (Fig. 2 S3). The method was first applied by Bergmann et al. in *A. nidulans* where the cluster-specific TF was integrated ectopically under the control of an inducible promoter. This led to the elucidation of the novel SMs aspyridones A and B and identification of the PKS-NRPS hybrid BGC responsible for the biosynthesis (Bergmann et al., 2007). Subsequently, several SMs and clusters have been linked and characterized using the same strategy; e.g. asperfuranone in *A. nidulans* (Chiang et al., 2009), a diterpene in *A. nidulans* (Bromann et al., 2012) and azaphilone in *A. niger* (Zabala et al., 2012). In *F. fujikuroi*, a pathway specific TF and the PKS backbone enzyme was overexpressed resulting in the production of 4 new SMs, fujikurins A-D (Janevska and Tudzynski, 2018; Von Bargen et al., 2015). Scopularide A and the responsible cluster was identified and verified by overexpression of the cluster specific TF in *Scopulariopsis brevicaulis* (Lukassen et al., 2015).

It is clear that the overexpression of a cluster-specific regulator can be a powerful strategy to induce SM production. However, it is not always straightforward. In a cluster containing two NRPS genes and a regulatory gene named *scpR* (for secondary metabolism cross-pathway regulator), the overexpression of *scpR* led not to the activation of the NRPSs as expected, but to the activation of the asperfuranone cluster located on another chromosome in *A. nidulans* (Bergmann et al., 2010). Ther conclusion was that cluster-specific TFs are not necessarily located within the cluster it is affecting, which thus adds another layer of complexity to BGC regulation.

Promoter exchange. When no cluster-specific TF is available, another option is to replace the promoters of all the genes in the BGC to force the expression of the cluster genes (Fig. 2 S4). This was done by Yeah et al. for the fellutamide B cluster in *A. nidulans*, where the promoters of six genes were replaced with the regulatable *alcA* promoter, with sequential gene manipulations by recycling a selectable marker (Yeh et al., 2016). A similar strategy was used in *A. nidulans* for the cluster responsible for a conidiophore pigment where the promoters of three genes (*ivoA-C*) were replaced with the *alcA* promoter (Sung et al., 2017).

2.1.2. Gene deletion or disruption strategies in active clusters

Cluster-specific gene deletions or disruptions. If a BGC is active under some known condition or it has been activated, it is possible to link the BGC to a SM. This is typically performed by deleting or disrupting genes encoding backbone synthases followed by subsequent metabolite profiling (Fig. 2 A1). By comparing the chemical spectrum of a wild type with the modified strain, it is possible to identify the SM missing in the modified strain and potentially identify intermediates in the biosynthetic pathway: this approach has been seen in numerous studies (e.g. Sanchez et al., 2011; Lo et al., 2012; Umemura et al., 2014; Wang et al., 2018). Elimination of gene function is by far the most used method for dissecting gene clusters. To this end, we note that in cases where SM enzymes exist in a complex, this approach may lead to accumulation of intermediates that are not directly reflecting the genetically impaired gene, but rather the activity of the complex as a whole since it may not form in the absence of one of its subunits. Hence, it may be preferable to include point mutations targeting catalytic essential residues in the SM enzymes, in cases where such residues can be identified, in the mutagenic dissection strategy.

The overall genetic strategy for BGC analysis is based on gene targeting or genetic screens. Historically gene targeting has been a cumbersome process since it, in most fungi, is inefficient as the desired strains typically were constructed with success rates below 10% due to the fact that most foreign DNA is inserted randomly into the genome by the non-homologous pathway (Krappmann, 2007). A higher efficiency can be achieved by using bipartite gene targeting substrates where the selectable marker is split into two non-functional but overlapping fragments (Fairhead et al., 1996; Catlett et al., 2003). Alternatively, gene-targeting efficiency can be dramatically elevated by eliminating genes in the non-homologous end-joining (NHEJ) pathway, i.e. genes that encode Ku70, Ku80 or DNA ligase IV (Ninomiya et al., 2004; Nayak et al., 2006). In such strains, gene targeting efficiency are typically higher than 90% (Krappmann, 2007). Hence, if many mutant strains are planned in a given species or strain background it may be a good investment to eliminate the NHEJ pathway prior to BGC mutagenesis. Importantly, to the best of our knowledge, no interference between defective NHEJ and SM production has been reported in the literature. However it has been shown that Ku deletions may lead to genetic instability (Meyer et al., 2007; Zhang et al., 2011).

Two genetic marker dependent strategies are commonly used for introducing genetic alterations in the genome. The first strategy is to perform one step gene disruptions/deletions, where a selectable marker is either inserted into the gene of interest to disrupt gene function; or where it simply replaces the gene of interest to create a gene deletion. This strategy only requires a single step, but if several genetic modification steps need to be performed, one may run out of selectable markers (Goosen et al., 1987; Daboussi et al., 1989). The second strategy is to use a two step loop-in/loop out method (Dunne and Oakley, 1988; Takahashi et al., 2012). This method requires selectable/counter selectable marker, e.g. *pyrG*, and is more time consuming. However, iterative gene targeting can be performed as it allows for marker re-cycling (Nielsen et al., 2006; Yeh et al., 2016). Other recyclable markers systems include the *beta-Rec/six* (Hartmann et al., 2010; Szewczyk et al., 2013) and the *Cre/loxP* (Krappmann et al., 2005;Forment et al., 2006; Florea et al., 2009;Steiger et al., 2011; Zhang et al., 2017) which are both based on site specific recombination (SSR), as reviewed by Krappmann (2014). In addition to produce gene disruptions and deletions, it can also be used to introduce specific point mutations. If nutritional markers are used for mutagenesis, it is highly recommended to restore the marker gene to wild-type as metabolic marker genes may alter the phenotype of the strain (Lay et al., 1998; Çakar et al., 1999; Pronk, 2002; Nielsen et al., 2007). It is further generally good practice to restore the phenotype after a deletion by reinsertion of the deleted gene, to prove that the phenotype is not the effect of a random mutation.

The recent introduction of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology in fungi is expected to revolutionize fungal genetic engineering (Shi et al., 2017; Krappmann, 2017; Deng et al., 2017). CRISPR technologies are based on the fact that the specificity of the Cas9 nuclease can be easily programmed by a short RNA species that is embedded in the enzyme. Hence, specific DNA double stranded breaks can be introduced into genes of interest to stimulate mutations created by error prone non-homologous end-joining or to stimulate gene targeting into that locus when a repair template is provided using homologous recombination (Nødvig et al., 2015; Zheng et al., 2018). Creating two double stranded breaks have been shown to increase the efficiency and making it possible to delete large regions (up to 48 kb region) (Zheng et al., 2018). A major advantage of the CRISPR technology is that it, in principle, can be used directly in a new species where little or no genetic tools are available and it is possible to perform all the classical gene-targeting tricks, even in a multiplexing setup, with no need for a selectable marker (Nødvig et al., 2018). The CRISPR-Cas9 system has successfully been established in a variety of fungal species including *Neurospora crassa* (Matsu-ura et al., 2015),

Trichoderma reesei (Liu et al., 2015), *Ustilago maydis* (Schuster et al., 2016), *Pyricularia oryzae* (Arazoe et al., 2015), *Candida albicans* (Vyas et al., 2015), *Nodulisporium* (Zheng et al., 2017) and several *Aspergillus species* (Nødvig et al., 2015;Fuller et al., 2015; Katayama et al., 2016;Nødvig et al., 2018) among others and more are constantly added. Moreover, the need for complex gene-targeting substrate construction, which is required for conventional gene targeting, is reduced to a minimum as simple oligonucleotides can be used to introduce gene deletions or specific point-mutations (Nødvig et al., 2018). It is important to stress that the methods listed above can be used in combination to further facilitate specific genetic engineering.

Comparisons of metabolite profiles obtained with mutant and reference strains may reveal HPLC signals that disappear in mutant strains, indicating that they represent the relevant SMs (Fig. 2). For strategies that can be used to analyze the often complex metabolite profiles, we refer the reader to (Nielsen et al., 2011; Nielsen and Larsen, 2015; Hubert et al., 2017). Importantly, false hits may occur in some experiments due to the complexity of the fungal metabolism or due to unpredictable genetic effects. Gene elimination experiments should therefore be accompanied by a complementation test where the mutated gene is returned to the mutant strain and, as a result production of the SM should be restored. Alternatively, or in addition, conclusions made from the analysis of synthase gene-deletion strains can be strengthened by deleting the tailoring enzyme genes of the BGC. In this set of mutant strains, the final SM should also be absent. As a bonus, metabolite profiling of the latter mutant strains may uncover intermediates for production of the final SM, hence, facilitating SM pathway elucidation.

The strategy of gene deletions (Fig. 2 A1) has been used in countless studies. In this paragraph, examples using variations of gene deletions are presented showing some of the many uses. Disruption of the *FUM5* gene combined with complementation studies revealed the involvement in fumonisin production in *Gibberella fujikuroi* (Proctor et al., 1999). Later *FUM6-FUM9* were identified in *Fusarium verticillioides*, analysed and disrupted showing that these genes are also involved in production of fumonisins (Seo et al., 2001). To investigate the red pigment aurofusarin produced by *Fusarium pseudograminearum* and *F. graminearum* Malz et al. created aurofusarin deficient mutants using first random mutagenesis to reveal a locus of interest including a PKS, *PKS12*. To confirm the *PKS12* gene involvement in aurofusarin production a targeted gene disruption was performed (Malz et al., 2005). The cyclopiazonic acid (CPA) biosynthetic genes were identified in *Aspergillus flavus* where three genes were disrupted and two of these mutants completely abolished CPA production (Chang et al., 2009). In *A. niger*, the *albA* gene was shown to be responsible for the production of pigments as well as naphtho-γ-pyrones through a deletion study. This helped identifying the link between SM and genes. In addition, it created a useful strain for further analysis since some of the main SMs produced by *A. niger* are disposed of, resulting in a more clean background strain (Chiang et al., 2011). Similarly, eight of the most highly expressed BGCs were deleted in *A. nidulans* resulting in a strain of low SM background allowing for the identification and characterization of aspercryptin and the biosynthetic genes (Chiang et al., 2016). In some cases gene deletion or disruption has turned out not to be possible and RNA silencing has been used as an alternative. This method was used in a study identifying the cytochalasin gene cluster in *Penicillium expansum* (Schumann and Hertweck, 2007).

Gene deletion libraries. A further sophistication of the deletion strategy focusing on one BGC of interest is the generation of deletion libraries (Fig. 2 A2). Gene deletion libraries can be generated in a number of ways, such as transposon disruptions, UV or chemical mutagenesis, or single-gene deletion libraries. Screening these afterwards for the absence of the compound of interest is a powerful approach.

A deletion library was created in *Gibberella zeae*, where 15 PKSs were disrupted, following known SMs such as zearalenone, aurofusarin, fusarin C and the black perithecial pigment were linked to specific genes (Gaffoor et al., 2005). In a similar study in *A. nidulans* each of the 32 predicted PKSs were deleted and the mutants were grown in various media and compared with the reference. This way they were able to identify the PKSs involved in arugosins, violaceol, austinol and dehydroaustinol biosynthesis (Nielsen et al., 2011). Another deletion library focused on the regulatory genes in *A. nidulans*, where 128 kinases were deleted. In this study it was observed that the secondary metabolism was affected in several of the mutants (De Souza et al., 2013). Yaegashi et al. screened this knockout library for changes in secondary metabolism. They found the SM aspernidine A and identified the secondary metabolite genes (*pkfA-F*) in *A. nidulans* (Yaegashi et al., 2013). Using panels of deletion mutants in the manner presented above has a great potential for future studies, given the developments in efficient genome editing technologies for fungi sparked by the CRISPR-Cas9 technologies (Nødvig et al., 2015).

2.1.3. Future possibilities in native strategies

With the advancement of molecular tools such as CRISPR-Cas9 in fungi (Nødvig et al., 2015), many of the native strategies will be applicable in more non-model species. Another future possibility based on the CRISPR-Cas system is the development of synthetic TFs where a deficient Cas9 can be fused to an effector domain thus inducing transcription of specific genes which has already been applied in yeast (Farzadfard et al., 2013).

2.2. Heterologous strategies

Heterologous expression strategies can be applied if the cluster of interest is found in a species which is difficult or impossible to propagate in the laboratory or if it is not easily genetically manipulated (Fig. 1). In addition it may often serve as an attractive tool to deliver insights that may complement those obtained with the native host.

The first thing to consider with heterologous expression is which host to select. This choice depends on many factors. We will here focus on three categories of hosts; bacteria, yeast, and filamentous fungi. The second thing to consider is the construct and how comprehensive the heterologous expression should be, including only backbone synthase, promoter swap of a TF, or engineering of the whole cluster with new promoters etc. for all genes in the cluster. Which construct to use depends on the cluster and the aim. In the following section, the three categories of hosts are examined followed by a section covering the expression construct.

2.2.1. Selection of host for heterologous gene expression

In choosing a host for expression of eukaryotic pathways, there are several factors to consider: (1) The amount of genetic optimization needed to express the genes – codon optimization, ability to process introns, change of promoter and terminator. (2) The natural ability to produce SMs, e.g. the availability of precursors and capability of post-translational modification to create active biosynthetic enzymes. (3) The chemical background — the amount of compounds produced affecting the chemical analysis. (4) The toxicity of the compound produced and the coping mechanism used in the natural producer (transporters, detoxification or resistance genes Keller, 2015) which should be included in a heterologous expression host as well. Here we will go through each of the three categories of host we consider, showing examples of their uses along with advantages and disadvantages of each.

Bacterial host. Escherichia coli is mainly used as a prokaryotic host due to the strong engineering toolbox and fast growth. Significant engineering of *E. coli* is needed in order to be able to produce fungal secondary metabolites since it does not naturally produce these compounds. *E. coli* is therefore predominantly used for *in vitro* investigation and characterization of specific enzymes in the pathway.

An early example of the use of bacterial host is the successful expression of the 6-methylsalicylic acid synthase gene from *Penicillium patulum* in *E. coli* which thereby produced 6-methylsalicylic acid

(Kealey et al., 1998). In a more recent study, the gene encoding the BbBEAS nonribosomal peptide synthetase isolated from *B. bassiana* was heterologously expressed in *E. coli* which was then able to produce beauvericin when the precursor D-Hiv was added (Heneghan et al., 2010; Xu et al., 2008).

Several of the tropolone biosynthetic genes from *Talaromyces stipitatus* were expressed in *E. coli* and purified for*in vitro*analysis thus verifying the expected biosynthetic pathway (Davison et al., 2012). The PKS4 protein from *Gibberella fujikuroi* was expressed in *E. coli*, purified and*in vitro*analysis was performed showing a functional enzyme producing the secondary metabolite SMA76a (Ma et al., 2007). Similarly the LovD protein from lovastatin biosynthetic cluster was expressed in *E. coli*, purified and analyzed*in vitro*(Xie et al., 2006). In an investigation of the basidiomycete *Coprinus cinereus*, six sesquiterpene synthases were expressed in *E. coli* and 5 of the products were identified directly from *E. coli* cultures using GC-MS (Agger et al., 2009). Using the same approach 11 sesquiterpene synthetase from *Omphalotus olearus* were characterized by heterologous expression in *E. coli* (Wawrzyn et al., 2012). In the investigation of the azaphilone gene cluster the gene *AzaH* (a FAD-dependent monooxygenase) was expressed in *E. coli* in order to make *in vitro* assays of the protein and investigate its function in azaphilone biosynthesis (Zabala et al., 2012).

E. coli has several advantages as a host for heterologous expression; (1) easy to cultivate and fast growing (2) a well-developed molecular toolbox (3) a well-understood primary metabolism and (4) the absence of endogenous secondary metabolite pathways thus limiting the risk of cross-talk and interference with native proteins (Gao et al., 2010; Pfeifer and Khosla, 2001).

There are however also several challenges when heterologously expressing fungal secondary metabolite genes in bacterial hosts. The challenges include: (1) inability of bacteria to process eukaryotic introns which thus have to be eliminated; (2) codon bias can cause problems in expression; (3) correctly folding of the synthesized proteins; (4) required post-translational phosphopantetheinylation/modifications; (5) potential anti-bacterial properties/toxicity of the product; and (6) availability of the precursors (Alberti et al., 2017; Gao et al., 2010; Heneghan et al., 2010). Bacterial hosts are therefore most often used for *in vitro* enzyme analysis of a specific biosynthetic protein.

Yeast as a heterologous host. The second host we will examine is yeast, in particular *Saccharomyces cerevisiae*. In the following we will go through examples of uses and end with a summary of advantages and challenges.

The team exploring the 6-methylsalicylic acid production in *E. coli* also investigated it in *S. cerevisiae* (Kealey et al., 1998). The synthase 6-methylsalicylic acid from *P. patulum* was expressed in a *S. cerevisiae* strain including a heterologous phosphopantetheinyl transferase which creates the active holo PKS from the apo-PKS (Lambalot et al., 1996; Kealey et al., 1998). From this strain, 6-methylsalicylic acid was produced and the amount was twice as high as in the native species and much higher than in *E. coli* (Kealey et al., 1998), hence a case where an eukaryotic host performed superior compared to *E. coli*.

The lovastatin nonaketide synthase LovB from *A. terreus* was expressed in an engineered strain of *S. cerevisiae* containing the a phosphopantetheinyl transferase gene *npgA* from *A. nidulans* (Mootz et al., 2002), in order to perform in-depth *in vitro* investigation of the catalytic function and mechanism (Ma et al., 2009), showing that yeast can be used to elucidate specific steps of biosynthetic pathways.

Ishiuchi et al. engineered a *S. cerevisiae* strain to include *matB* (malonyl-CoA synthetase) and *npgA* and successfully used this for expression of five PKSs and one NRPS and characterization of the produced SMs (Ishiuchi et al., 2012). Several other studies have used optimized versions of *S. cerevisiae* for heterologous expression of synthase genes, for identification of the products and characterization of the mechanisms, including identification of 10,11-Dehydro-curvularin and characterization of a mechanism for aryl-aldehyde (Wang et al., 2014; Xu et al., 2013).

In addition to single synthase genes whole clusters have also been expressed in *S. cerevisiae*. The biosynthetic genes from the hypothemycin gene cluster from *Hypomyces subiculosus* were for instance expressed in a PKS -optimized yeast strain (Kealey et al., 1998; Reeves et al., 2008) and based on these experiments, it was possible to propose a biosynthetic pathway (Reeves et al., 2008). In another study by Rugbjerg et al. three biosynthetic genes from *Fusarium graminearum* were co-expressed with the *npgA* gene from *A. fumigatus* resulting in the production of Rubrofusarin (Rugbjerg et al., 2013).

In a recent study, Harvey et al. developed HEx (Heterologous EXpression) synthetic biology platform for fast and scalable expression of fungal biosynthetic genes and their encoded metabolites in *S. cerevisiae* (Harvey et al., 2018). In this study 41 BGCs from diverse ascomycete and basidiomycete fungal species were expressed in *S. cerevisiae* and 54% resulted in SMs not natively found in yeast (Harvey et al., 2018). This platform brings the method of heterologous expression in yeast a big step forward and potentially opens the door to discovery of many natural products.

Using *S. cerevisiae* as a heterologous host has many advantages, some of which are similar to those mentioned for *E. coli*, but there are additional advantages to using *S. cerevisiae*: (1) *S. cerevisiae* is a unicellular organism, easy to culture and it grows faster than most filamentous fungi; (2) Powerful genetic tools have been developed for protein expression and pathway construction, including highly efficient homologous recombination; (3) Native secondary metabolism is very limited in *S. cerevisiae* thus minimizing the background and potential cross-talk (Siddiqui et al., 2012); (4) The building blocks for polyketide synthesis such as acetyl-CoA and malonyl-CoA plus cofactors such as NADPH and S-adenosylmethionine are naturally produced in yeast; (5) Lastly yeast also belongs to the fungal kingdom and it can typically produce tailoring enzymes and support correct folding (Bond et al., 2016; Alberti et al., 2017).

The challenges of using *S. cerevisiae* as a heterologous host include: (1) A heterologous gene is required for activation of the synthase such as the *npgA* gene from *A. nidulans* (Mootz et al., 2002); (2) *S. cerevisiae* has different and few introns which can cause problems in mRNA splicing (Kupfer et al., 2004); (3) In yeast codon usage is biased towards AT which can cause problems if the gene of interest is GC rich (Mutka et al., 2006); A low or even lacking production of required precursors and building blocks (Kealey et al., 1998; Mutka et al., 2006); (4) Lack of compartmentalization which might be important for SM production (Roze et al., 2011); (5) Lastly there is a risk of toxicity of the produced SM.

Filamentous fungi as heterologous host. Many of the challenges seen for yeast and bacterial hosts can be overcome by using filamentous fungal hosts. The model fungus *Aspergillus nidulans* is often used, because it has a well developed genetic toolbox, *A. niger* has been shown to be a highly efficient host (Boecker et al., 2018) and *A. oryzae* which has a limited endogenous secondary metabolism is often employed.

Initially many of the studies of heterologous expression were based on a single gene, often the backbone enzyme. One example is the study of *albA* from *A. fumigatus* which is involved in conidial pigment biosynthesis and it was heterologously expressed in *A. oryzae* to show that the PKS is a naphthopyrone synthase (expected based on the sequence similarity) and not a tetrahydroxynaphtalene synthase (expected from the color) (Watanabe et al., 2000). The drawback of single gene studies is that most often they do not give the final secondary metabolite or elucidate the biosynthetic pathway.

Other studies have expressed whole clusters in heterologous hosts. Smith et al. was one of the first to do this: they cloned the penicillin BGC from *Penicillium chrysogenum* on to a cosmid vector, transferred it to *Neurospora crassa* and *A. niger* and showed that penicillin was produced (Smith et al., 1990). This approach however relies on the native promoters functioning in the new host and correct mRNA processing.

One method of circumventing this dependency is to place the TF under a strong promoter. This approach was seen in a study of a cryptic

polyketide cluster from *Trichophyton tonsurans* where four biosynthetic genes of the cluster were expressed from their own promoters, and only the cluster-specific TF promoter was replaced with the strong *A. nidulans gpdA* promoter (Yin et al., 2013). A neat detail in the design of this study was that the cluster was inserted in the *wA* locus of *A. nidulans* encoding a pigment PKS, facilitating the screening of correct recombination. Another successful heterologous expression system was made based on regulatory elements from the *Aspergillus terreus* terrein gene cluster (Gressler et al., 2015; Brandt et al., 2017).

Many studies have been conducted investigating BGCs by heterologous expression, including pyripyropene from *A. fumigatus* expressed in *A. oryzae* (Itoh et al., 2010), the citrinin cluster from *Monascus purpureus* expressed in *A. oryzae* (Sakai et al., 2008) and the *Pfma* cluster from *Pestalotiopsis fici* expressed in *A. nidulans* synthesizing the melanin 8-dihydroxy- naphthalene (DHN) (Zhang et al., 2017) to mention a few.

Heterologous expression in filamentous fungi has some challenges, the host can be affected by the inserted cluster causing a changed metabolite profile or cross-talk between the inserted cluster and native clusters can arise which makes it difficult to identify the correct new SM. This was illustrated by a study expressing a polyketide gene cluster originating from a fungal endophyte, in *Fusarium verticillioides* (Xie et al., 2011), where the main product identified was fusaric acid, which is a mycotoxin normally found in *Fusarium* species.

There are several studies optimizing heterologous hosts to make the strategy more effective. Chiang et al. developed an optimized heterologous expression system in *A. nidulans* (Chiang et al., 2013). Their first step was to delete native BGCs (of sterigmatocystin, emericellamide, orsellinic acid/F9775A,B, asperfuranone, monodictyphenone, and terrequinone) in order to reduce the SM background and facilitate detection of novel products plus to increase the pool of pre-cursors for the desired products. Next, they developed a method for heterologous expression of biosynthetic genes using a system of a recyclable marker thus permitting the expression of entire clusters, which was shown for the asperfuranone cluster from *A. terreus*.

Using fungal artificial chromosomes (FACs) and metabolomic scoring (MS) it is possible to scale up analyses, which was demonstrated by investigating 56 BGCs originating from *A. terreus, A. aculeatus* and *A. wentii* in *A. nidulans* (Clevenger et al., 2017). In the study, 17 SMs produced by 15 different FACs were detected. In a subsequent study the FAC-MS method was used to elucidate the biosynthesis of acu-dioxomorpholine (Robey et al., 2018).

The advantages of using a filamentous fungus as host includes; (1) The genetic systems are generally compatible correctly translation folding and post-translational modifying the inserted gene(s) hence obviating the need for codon optimization, intron removal etc. (2) The secondary metabolite machinery is present, making most common precursors available. The downside is that the complex chemical background can make the chemical analysis difficult and cause cross-chemistry making it complicated to identify the SM produced by the inserted genes. An additional disadvantage is that it is time-consuming using filamentous fungi as hosts since they are challenging to engineer (mainly due to the possibility of heterokaryons) and slower growing than many yeasts and bacteria.

2.2.2. Design of DNA constructs

Besides selecting the host for heterologous expression, another thing to consider is the construct as mentioned earlier. Expressing a gene heterologously either depends on the host having similar promoters and terminators and mRNA transcript processing or it requires extensive engineering of the gene. The design of the construct depends on the composition of the gene cluster, the host and the aim. Here we have divided it into three main strategies: (1) Expression of the synthase/synthetase, (2) Inserting the entire cluster, potentially with engineering of the TF and (3) Engineering of the whole cluster including new promoter etc. for all genes.

Strategy 1 — only expressing the synthase of a cluster is often used in initial studies of cryptic BGCs. This method is used to investigate the core structure of the secondary metabolite (SM) to give an initial indication of what structure is produced and can also be used in screening studies. The strategy was used in the study of six sesquiterpene synthases from mushroom-forming fungi (*Agaricomycetes*) where the sesquiterpene synthases were expressed in *E. coli* and/or *S. cerevisiae*, thereby characterizing the enzymes and identifying the major sesquiterpene hydrocarbons produced (Agger et al., 2009). Ishiuchi et al. expressed five PKSs and an NRPS in *S. cerevisiae* and identified the corresponding natural products (Ishiuchi et al., 2012). Likewise two polyketide synthases responsible for cladosporin production were expressed in *S. cerevisiae* to confirm the involvement in cladosporin production and understand the mechanism and biosynthesis (Cochrane et al., 2016). Munawar et al. expressed a nonribosomal peptide synthetase from *Fusarium sacchari* in *A. oryzae* and showed that it is responsible for producing the siderophore ferrirhodin (Munawar et al., 2013). There are examples where this strategy is not suitable such as clusters containing trans acting enoyl reductase (ER) domains (Ames et al., 2012; Simpson, 2014; Ugai et al., 2015).

Expressing only the synthase has the advantage that it is most often only one gene, making it easier and less labor-intensive to exchange promoter and occasionally terminator. The disadvantage is that it does not give the final SM, but it gives the core structure of the SM and the initial step in the biosynthesis.

Strategy 2 — Insertion of the entire BGC only modifying a cluster-specific TF, if present. This strategy allows analysis of the entire biosynthetic cluster and the final product with minimal engineering but requires compatible cellular machinery or a TF. It is possible to successfully transfer an entire BGC to another species without modification however this requires that the transcriptional and translational machinery are compatible between the donor and host. One example is the citrinin gene cluster from *Monascus purpureus*, which was successfully expressed in *A. oryzae* without modification of the BGC (Sakai et al., 2008). It is often difficult to know if the donor and host are compatible in advance and even when successful the amount of SMs produced are often low. If the cluster contains a specific TF, it is possible to only exchange the promoter of the TF and get increased expression of the rest of the cluster; this is similar to the strategy of activating silent gene clusters in Section 2.1.1. The citrinin cluster also contains a TF and when this was overexpressed in *A. oryzae*, the production increased 400-fold compared to insertion without modification (Sakai et al., 2008). Similarly, the geodin cluster from *A. terreus* was successfully expressed in *A. nidulans* by replacing the native promoter of the TF with a strong constitutive promoter (Nielsen et al., 2013).

The advantage of this strategy is that it produces the final SM of the cluster and it requires minimal engineering of the cluster. The disadvantage is that it requires compatible cellular machinery of the native and heterologous host and/or the presence of a cluster-specific TF.

Strategy 3 — engineering of the entire cluster exchanging all the promoters, can be necessary if the cluster of interest does not contain a TF. Four biosynthetic genes from *Phoma betae* were expressed in *A. oryzae* in a vector-based approach using the starch-inducible promoter/terminator from the *amyB* gene thereby producing aphidicolin (Fujii et al., 2011). Bailey et al. successfully expressed seven biosynthetic genes from the basidiomycete *Clitopilus passeckerianus* in *A. oryzae* using constitutive *A. oryzae* promoters thereby producing pleuromutilin (Bailey et al., 2016). An alternative to swapping all the promoters in the cluster is using polycistronic gene expression which has been successfully applied in *A. niger* in a combination with the Tet-on system for production of enniatin (Meyer et al., 2011; Schuetze and Meyer, 2017).

The advantage of engineering the entire cluster is that there are no requirements or restrictions as to which clusters can be investigated. The drawback is that it is a more labor-intensive method and it can be difficult to know where the cluster borders are. If one has transcriptome data available, there are established methods for using this to predict cluster boundaries (Andersen et al., 2012; Umemura et al., 2013; Vesth

et al., 2016). With the ever increasing numbers of fungal transcriptome experiments (see e.g. Schäpe et al., 2019), these methods only become more applicable in the future. There are several excellent reviews on the subject of heterologous expression, for further reading please refer to Alberti et al. (2017),Anyaogu and Mortensen (2015), and Lazarus et al. (2014).

Future perspectives. As the price of synthesis of long stretches of DNA keeps dropping due to improved chemistries and synthesis methods, novel strategies are emerging, making it possible to circumvent several time consuming cloning steps (intron removal, promoter swap, codon optimization etc.) and thereby easier to investigate selected clusters or screen more clusters. In particular, the strategy of promoter swapping of all genes in a cluster becomes much more feasible when using synthetic DNA.

3. Part II: *In silico* strategies associating secondary metabolites to biosynthetic gene clusters based on whole genome sequences

The aim of the strategies presented in Part II is to identify a biosynthetic gene cluster (BGC) responsible for producing a specific secondary metabolite. The selected secondary metabolite is thus the starting point, it can be a characterized compound with known chemical structure or it can simply be a new signal in a chromatogram. Alternatively, the starting points can be already characterized SMs with identified biosynthetic gene clusters, which are used to search for similar BGCs in a genome and hence assess the potential of SM production of an organism. If an interesting SM has been identified in a certain species, there are several methods for identifying the responsible BGC. Here we have divided it into three main approaches (1) Homology search (2) Retro biosynthesis and (3) Comparative genomics (Fig. 3). Which strategy to use essentially depends on the initial knowledge and as seen in the strategies in Part I, a combination of several strategies is often needed to identify and verify the BGC.

A prerequisite for all the strategies mentioned here is that whole genome sequences are available for the producing organism(s). These methods have thus only been made possible in the post-genomic era. As whole genome sequences are becoming more and more attainable, the use of these strategies will surely only increase. It is important to note that the results of these strategies are putative and requires some experimental verification following the identification.

3.1. Homology search

In strategy 1, based on homology search, a biosynthetic genes cluster producing a specific compound is identified by using a characterized biosynthetic gene cluster producing the same or a similar compound (Fig. 3, Strategy 1). The known biosynthetic cluster genes are used as query to search for similar genes in the genome of the organism producing the selected compound of interest. In general the most important genes are the synthase/synthetase responsible for producing the backbone of the SM. This strategy only works if a similar SM and BGC have been characterized previously, it can thereby only be used to find known clusters or derivatives, but not truly new SMs. Homology search is an extremely powerful and highly employed approach for coupling clusters and SMs especially in the investigation of newly sequenced genomes. As the number of whole genome sequences increases and the algorithms aiding in the predictions are improved, this method is likely to expand.

A derivative of this strategy (genetic dereplication Theobald et al., 2018) has a wider scope, not focusing on a single SM, but instead

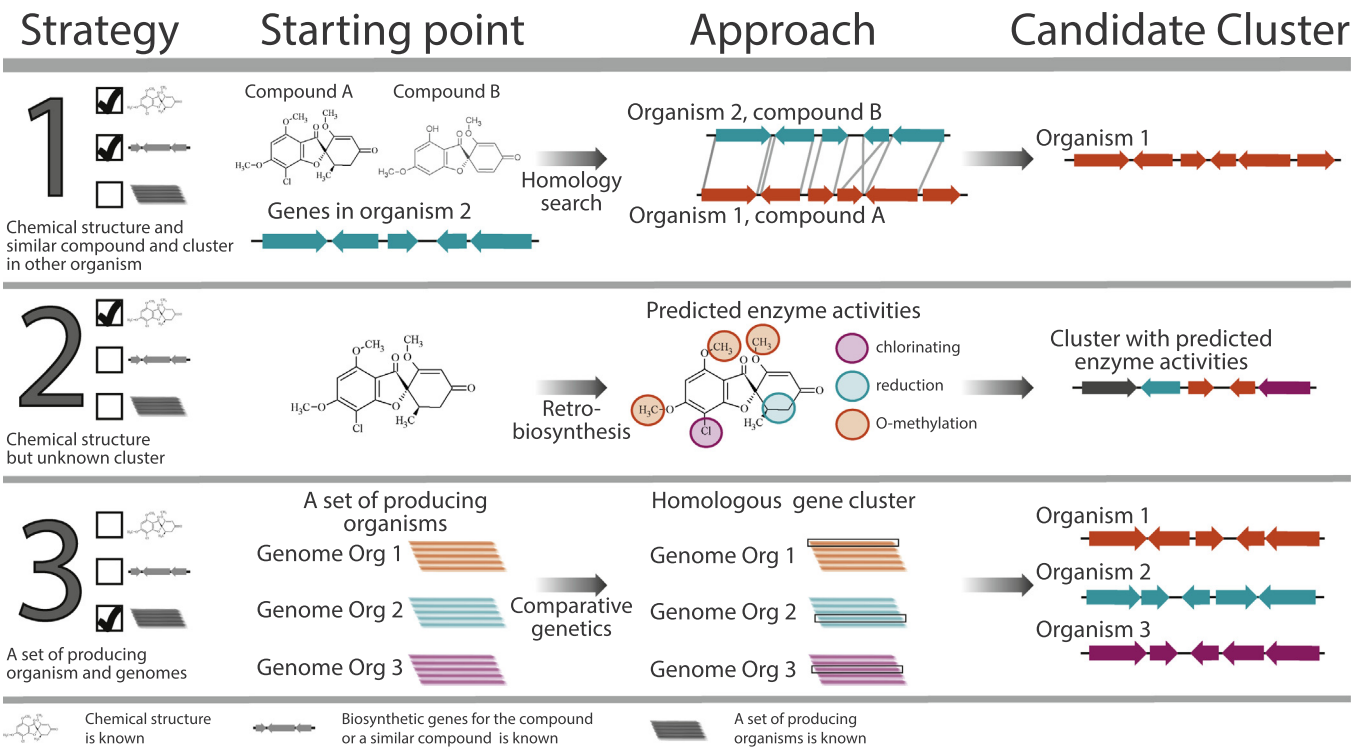

Fig. 3. Three strategies for linking secondary metabolite to biosynthetic gene cluster using whole genome sequences. (1) Homology search — starting with a known compound produced by organisms A and the same or similar compound produced by organism B where the cluster has been identified it is possible to use the known cluster from organism B to search for a similar cluster in the genome of organism A and thereby identify the cluster of interest. (2) Retro-biosynthesis — starting with a known compound but no similar clusters identified it is possible the predict the enzyme activities needed to produce the compound (backbone and tailoring enzymes) and from these predictions find putative clusters matching the requirements in the genome. (3) Comparative genomics — starting with a set of organisms where some produces the compound of interest and some do not, it is possible to identify homologous gene clusters in the producing and filter based on no homologs in the non-producing and thereby identify candidate gene clusters. All the strategies lead to the identification of a candidate cluster which then have to undergo functional verification.

identifying homologs of all characterized SMs and BGCs. Hence all known SMs and their BGCs can be used as query to search for homologous clusters in a newly sequenced genomes. This way it is possible to predict what kind of SMs the organism potentially can produce.

An example of the homology search strategy is the ochratoxin gene cluster that was identified in *A. carbonarius*, using homology search of the ochratoxin cluster predicted in *A. niger*. The following deletion of a PKS in the predicted cluster eliminated all production of ochratoxin confirming the biosynthetic role (Gallo et al., 2014). The method of homology search can also be used to find the putative clusters for similar SMs that could use parts of the same biosynthetic pathway. This was shown for in the identification of the novofumigatonin cluster in *A. novofumigatus* which was identified based on homology to another meroterpenoid, the terretonin cluster from *A. terreus* (Kjærbølling et al., 2018; Matsuda et al., 2018).

Using the derivative of homology search (genetic dereplication), it is also possible to investigate the secondary metabolite potential of a newly sequenced species. The secondary metabolism was investigated in the wheat pathogen *Zymoseptoria tritici* using MultiGene Basic Local Alignment Search Tools (BLAST) (Medema et al., 2013) to identify known clusters. This strategy revealed a cluster similar to the ferrichrome-A biosynthetic locus from a maize pathogen, *Ustilago maydis*. Putative clusters for carotenoid/opsin, an epipolythiodioxopiperazine, fumonisin and AM-toxin were also identified (Cairns and Meyer, 2017). After whole genome sequencing of *Penicillium griseofulvum*, various cluster products were predicted based on homology to known clusters from the MIBiG database (Medema et al., 2015). This revealed that *P. griseofulvum* has putative gene clusters for patulin, roquefortine C/meleagrin, griseofulvin, penicillin, cyclopiazonic acid, yanuthone D and chanoclavine I (Banani et al., 2016). Similarly, the genome of *A. ustus* — a rare human pathogen — was searched for the presence of known clusters and several commonly known aspergillus SM clusters were identified; monodictyphenone, sterigmatocystin, emericellamide, ferricrocin and asperthecin. In addition a putative cluster for viridicatumtoxin not previously identified in Aspergillus species was also identified in *Aspergillus ustus* (Pi et al., 2015). A homology search approach (based on ClusterBLAST module embedded within FungiSMASH Blin et al., 2017) was also applied in the investigation of the lichen, *Cladonia uncialis*. This revealed genes homologous to the lichen metabolite grayanic acid from *C. grayi* as well as clusters likely encoding fungal SMs not identified in lichens before such as patulin and betaenones AC (Bertrand et al., 2018).

3.2. Retro in silico biosynthesis

In strategy 2, based on *in silico* retro-biosynthesis, a biosynthetic genes cluster producing a specific compound is identified by deducing what enzyme activities are needed to produce the compound and searching the genome for those activities (Fig. 3). The stating point is thus the chemically characterized secondary metabolite combined with knowledge of secondary metabolite biosynthesis and an annotated whole genome sequence.

The anticancer lipopeptide, scopulariide A, is produced by a marine-derived *Scopulariopsis brevicaulis* and the chemical structure consists of a reduced carbon chain coupled to five amino acids. The SM is structurally related to emericellamide A and W493-B from *A. nidulans* and *F. pseudograminearum*, respectively. After the sequencing of *S. brevicaulis*, Lukassen et al. wanted to identify the cluster responsible for scopulariide A production in order to optimize the production. This was done primarily based on a retro-biosynthetic approach supported by homologous comparisons. By combining the knowledge of the structure with predicted BGCs it was possible to identify genes encoding the SM, a NRPS with five modules and a reducing PKS. The identified genes also showed homology to the clusters for the structurally related SMs emericellamide A and W493-B. The putative cluster included a predicted TF. To further support the prediction and to improve the production of scopulariide A the TF was overexpressed which significantly increased the production of scopulariide A thus indirectly verifying the prediction (Lukassen et al., 2015).

A retro-biosynthesis-based approach was also used in the identification of the putative usnic acid cluster in the lichen fungal partner of *Cladonia uncialis* (Abdel-Hameed et al., 2016). After de novo sequencing of *C. uncialis*, the genome was mined for PKS genes. From the structure of usnic acid and an earlier labelling experiment, it was suggested that usinic acid biosynthesis requires a non-reducing PKS including a methylation domain and a terminal Claisen cyclase (CLC) domain plus an oxidative tailoring enzyme, most likely a cytochrome P450. Based on this information, the predicted PKS clusters were screened, and only one matched the requirements. To further support the prediction, transcriptional analysis of the genes was performed under conditions where only usnic acid was produced, which confirmed that the identified genes were transcriptionally active.

Khater et al. have attempted to develop a computational protocol based on the concept of retro-biosynthesis to reconstruct biosynthetic pathways of polyketides and nonribosomal peptides (Khater et al., 2016). The aim is to predict the enzymes and the gene functions involved in the biosynthesis of a certain SM and thus be able to predict and identify the responsible BGC in an automated manner. The developed approach was tested based on 78 experimentally characterized secondary metabolites (51 PKS/HYBRID, 27 NRPS), here it was able to predict 37% correctly, 13% with minor errors, 24% partially correct and 26% incorrectly. The predictive methods are still in the early stage and need more knowledge of the mechanisms behind the secondary metabolite production and development but it has the potential to become a very powerful tool in the future.

3.3. Comparative genomics

In strategy 3, based on comparative genomics, biosynthetic gene clusters producing a specific compound are identified by comparing the genomes of a set of organisms producing the compound of interest (Fig. 3). The starting point is thus a list of whole genome sequenced organisms producing the compound of interest, the exact chemical structure is not required. The species used could for instance be distantly related species producing the same SM or closely related species sharing a high degree of secondary metabolism, but not the SM of interest. This way the presence/absence pattern of the SM can be used to search for similar patterns of BGCs across the full genome sequence. This could be of interest when a bioactive compound of pharmaceutical interest is known to exist, but the biosynthetic pathway and the BGC is not. The advantage of this strategy is that no knowledge about the biosynthetic gene cluster is needed nor the chemical structure of the compound, only a set of organisms with a specific pattern of producing/not producing a specific compound and their genomes are required. One thing to be aware of is that non-producers could have silent clusters and therefore a chemical negative should not be taken as an absolute, but rather an indication. Currently this strategy has only been applied on a limited scale but as more and more species are sequenced, we expect that the comparative analysis will expand and more sophisticated methods will develop.

The identification of the viridicatumtoxin and griseofulvin gene clusters in *Penicillium aethiopicum* was accomplished using such comparative genomics strategies: *P. aethiopicum* was sequenced and compared to the *Penicillium chrysogenum* genome which is a closely related species but it does not produce the SMs of interest. This way 9 out of 30 predicted PKSs could be ruled out due to homology between them. To further narrow it down, retro-biosynthetic methods was used to identify the most likely kind of PKS and to the check that the surrounding genes match the expected tailoring enzymes. This way candidate clusters for both SMs were identified and these were verified by gene deletion and RNA silencing (Chooi et al., 2010).

The biosynthetic clusters of the (+)/(-)-notoamide,

paraherquamide and malbrancheamide pathways (all based on bicyclo [2.2.2]diazaoctane indole alkaloid core) were identified based on homology search and comparative genomics. The genomes of *A. versicolor* NRRL35600, *P. fellutanum* ATCC20841, and *M. aurantiaca* were sequenced and the (-) notoamide cluster known from *Aspergillus* sp. MF297-2 (Ding et al., 2010) was used to search for homologs in the newly sequenced species (Li et al., 2012). Comparison of the identified clusters led to the identification of genes responsible in the formation of the bicyclo[2.2.2]diazaoctane core along with specific enzymes responsible for specific differences in the chemical structures (Li et al., 2012).

The malformin BGC was also identified using comparative genomics in combination with retrobiosynthesis. Three *Aspergillus* section *Nigri* species are known to produce malformin and these were searched for predicted BGC clusters matching the functions expected based on retrobiosynthesis of the compound. This way a candidate cluster was identified and experimentally verified (Theobald et al., 2018).

Comparative genomics strategies are often used with a combination of retro-biosynthesis and/or homology search, again showing that a combination of strategies is most often needed to establish the links between SM and gene clusters.

4. Conclusion

Many strategies have been developed and employed in the quest to link secondary metabolites to their biosynthetic gene clusters and vice versa. As the technologies and tools continue to evolve and we gain a deeper understanding of the mechanisms behind secondary metabolite production the speed and efficiency of linking SM and clusters will increase.

We see three major areas of advancement in the near future. Firstly, molecular tools (such as CRISPR) are quickly developing, making it feasible to work with many different species and thereby making it possible to conduct analysis in the native species. We will therefore see more studies from non-model organisms. Secondly, as the price of de novo synthesis of DNA is rapidly decreasing heterologous expression of silent clusters will become easier, opening for larger high throughput screening studies. Thirdly, with the increasing number of whole genome sequences and knowledge of secondary metabolism more comparative genomics approaches will be used and advanced bioinformatic tools will emerge making more accurate and more advanced predictions.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.fgb.2019.06.001.

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